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Immunization of Aged Mice with a Pneumococcal Conjugate Vaccine Combined with an Unmethylated CpG-Containing Oligodeoxynucleotide Restores Defective Immunoglobulin G Antipolysaccharide Responses and Specific $CD4^+$-T-Cell Priming to Young Adult Levels

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Polysaccharide (PS)-protein conjugate vaccines, in contrast to purified PS vaccines, recruit $CD4^+$-T-cell help and restore defective PS-specific humoral immunity in the immature host. Surprisingly, in the immunocompromised, aged host, anti-PS responses to conjugate vaccines are typically no better than those elicited by purified PS vaccines. Although aging leads to defects in multiple immune cell types, diminished $CD4^+$-T-cell helper function has recently been shown to play a dominant role. We show that in response to immunization with purified pneumococcal capsular PS serotype 14 (PPS14) in saline, the T-cell-independent immunoglobulin G (IgG) anti-PPS14 response in aged mice was comparable to that in young mice. In contrast, the T-cell-dependent IgG anti-PPS14 response to a soluble conjugate of PPS14 and pneumococcal surface protein A (PspA) (PPS14-PspA) in saline was markedly defective. This was associated with defective priming of PspA-specific $CD4^+$ T cells. In contrast, immunization of aged mice with PPS14-PspA combined with an unmethylated CpG-containing oligodeoxynucleotide (CpG-ODN) restored IgG anti-PPS14 responses to young adult levels, which were substantially higher than those observed using purified PPS14. This was associated with enhanced PspA-specific $CD4^+$-T-cell priming. Similarly, intact *Streptococcus pneumoniae* capsular type 14, which contains Toll-like receptor (TLR) ligands, also induced substantial, though modestly reduced, T-cell-dependent (TD) IgG ant-PPS14 responses in aged mice. Spleen and peritoneal cells from aged and young adult mice made comparable levels of proinflammatory cytokines in response to CpG-ODN, although cells from aged mice secreted higher levels of interleukin-10. Collectively, these data suggest that inclusion of a TLR ligand, as an adjuvant, with a conjugate vaccine can correct defective TD IgG anti-PS responses in elderly patients by augmenting $CD4^+$-T-cell help.

The incidence of infections, including pneumococcal (Pn) pneumonia and sepsis, is significantly elevated in the elderly relative to young adults (37, 60). Defective innate immunity including diminished neutrophil and macrophage function may be partly responsible (8, 41, 55). Furthermore, the aged host elicits decreased amounts and avidity of antibody in response to various antiviral and antibacterial vaccines, including those for intact *Streptococcus pneumoniae* (7, 14, 38, 40). This is correlated with a reduced germinal center reaction and a consequent diminution in somatic hypermutation, affinity maturation (67), generation of long-lived plasma cells (36), and induction of B-cell memory (58) following immunization. Defective $CD4^+$-T-cell help appears to play an especially important role in the age-related decline of the adaptive immune response (19). Of note, Eaton et al. previously demonstrated that when $CD4^+$ T cells from young adult mice were transferred into aged mice, they induced B-cell proliferation and immunoglobulin G (IgG) production comparable to those seen upon similar transfer into young adult recipients (15). Exposure to inflammatory cytokines in vivo could restore the defective $CD4^+$-T-cell function in aged mice (20).

Pn polysaccharide (PS) (PPS) vaccine, which induces protective IgG responses in a T-cell-independent (TI) manner, is recommended for all elderly patients (>65 years of age) (11). However, the relative effectiveness of this vaccine in the elderly is currently a matter of controversy. Thus, one study reported some efficacy of the PPS vaccine in preventing pneumococcal bacteremia in the elderly (24) but not in preventing community-acquired pneumonia (23, 24). However, another study demonstrated that PPS vaccine could in fact reduce pneumonia in all cases, including patients with community-acquired pneumonia, elderly patients with chronic lung disease (39), and patients hospitalized long term (59). In contrast, a recent analysis of multiple published studies concluded that, overall, there is no statistical proof that PPS vaccines protect the elderly from pneumococcal infections (4, 32). Collectively, these data suggest that although PPS vaccine may have some usefulness in the elderly in certain clinical contexts, improved antipneumococcal vaccines for the elderly population are clearly warranted (42).

Covalent linkage of capsular PS to an immunogenic carrier protein converts the PS from a TI to T-cell-dependent (TD) antigen capable of eliciting $CD4^+$-T-cell help for anti-PS responses (17, 18). This results in a striking increase in PS-specific immunogenicity, particularly in the immature host (5, 44), where Ig responses to PS antigens are otherwise highly defective (53). Surprisingly, in adults, a variety of clinical studies have failed to show clear differences in immunogenicity between conjugate vaccine and corresponding unconjugated

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PS vaccine, especially in those who are immunocompromised, including the elderly (1, 42). The mechanism underlying this observation is unknown. An understanding of why conjugate vaccines fail to exhibit superior PS-specific immunogenicity over pneumococcal PS vaccines in the elderly host might suggest approaches for boosting anti-PS titers, and corresponding protective immunity, in response to conjugate vaccines in this at-risk population.

The age-related decline in $CD4^+$-T-cell function suggests one potential reason for why the TD Pn conjugate vaccine appears no more immunogenic than the TI PPS vaccine in the elderly. Thus, in this report, we measured Ig responses to both PPS and Pn conjugate in young adult and aged mice. We show that whereas young adult and aged mice made comparable anti-PPS responses to PPS antigens, aged mice were markedly defective in their anti-PPS response to Pn conjugate. This was associated with defective priming of specific $CD4^+$ T cells. Inclusion of an unmethylated CpG-containing oligodeoxynucleotide (CpG-ODN), a Toll-like receptor 9 (TLR9) ligand (21) that induced comparable levels of proinflammatory cytokines in immune cells from young adult and aged mice, restored the anti-PPS response to Pn conjugate in the aged mice, which is associated with enhanced $CD4^+$-T-cell priming. Collectively, these data suggest that the poor immunogenicity of conjugate vaccines in the elderly is due, at least in part, to defective $CD4^+$-T-cell help. These data also suggest that inclusion of a proinflammatory TLR ligand with Pn conjugate vaccine will boost $CD4^+$-T-cell help and improve protective humoral immunity in the elderly above that seen with the currently used PPS vaccine.

MATERIALS AND METHODS

Mice. Young adult female BALB/c mice were obtained from the National Cancer Institute (Frederick, MD) and were used between 8 and 12 weeks of age. Aged female BALB/c mice were obtained from the National Institute of Aging (Bethesda, MD) and were used between 16 and 18 months of age. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council (38a), and were approved by the Uniformed Services University of the Health Sciences Institutional Animal Use and Care Committee.

Reagents. Recombinant PspA was expressed in *Saccharomyces cerevisiae* BJ3505 as a His_6-tagged fusion protein and purified by nickel-nitrilotriacetic acid affinity chromatography (62). Phosphorylcholine (PC)-keyhole limpet hemocyanin (KLH) was synthesized as described previously (62). The resulting conjugate had a substitution degree of 19 PC molecules/KLH molecule. Purified pneumococcal capsular polysaccharide type 3 (PPS3) and type 14 (PPS14) were purchased from the American Type Culture Collection (Manassas, VA). Purified pneumococcal cell wall C polysaccharide (C-PS) was purchased from Statens Serum Institut (Copenhagen, Denmark).

Preparation and immunization with *S. pneumoniae* capsular type 14. A frozen stock of *S. pneumoniae* capsular type 14 (Pn14) was thawed and subcultured on BBL premade blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Todd Hewitt broth (Becton Dickinson, Sparks, MD) to mid-log phase, collected, and heat killed by incubation at 60°C for 1 h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with phosphate-buffered saline (PBS) to give an absorbance reading at 650 nm of 0.6, which corresponded to 10^9 CFU/ml. Bacteria were then aliquoted at 10^{10} CFU/ml and frozen at −80°C until their use as an antigen for mouse immunizations. Mice were immunized intraperitoneally (i.p.) with 2×10^8 CFU of heat-killed bacteria in 250 μl of PBS. The Pn14 stock was tested for endotoxin using the *Limulus* amebocyte lysate assay (QCL-1000) from BioWhittaker (Walkersville, MD). This assay demonstrated that mice injected with 2×10^8 CFU equivalents of Pn14 receive <20 pg of endotoxin (if at all). Serum samples for measurement of antigen-specific IgM and IgG titers were prepared from blood obtained through the tail vein.

Preparation and immunization with PPS14-PspA. A soluble conjugate consisting of PPS14 covalently linked to PspA (PPS14-PspA) was synthesized as described previously (26). Young and aged mice were immunized i.p. with 1 μg each of soluble PPS14-PspA conjugate either in saline or adsorbed on 13 μg of alum (Allhydrogel, 2%; Brenntag Biosector, Denmark) mixed with 25 μg of a 30-mer CpG-ODN (47). Mice were boosted in a similar fashion on day 11. This suboptimal dose of alum, when used in the absence of CpG-ODN, exhibited no adjuvanting effect on the Ig response to conjugate, relative to conjugate suspended in saline (data not shown), and was used simply to facilitate the association of the conjugate with the CpG-ODN.

Measurement of serum antigen-specific Ig isotype titers. Immulon 4 enzyme-linked immunosorbent assay (ELISA) plates (Dynex Technologies, Inc., Chantilly, VA) were coated (50 μl/well) with PC-KLH (5 μg/ml), PPS3 or PPS14 (5 μg/ml), or PspA (5 μg/ml) in PBS for 1 h at 37°C or overnight at 4°C. Plates were washed three times with PBS plus 0.1% Tween 20 and were blocked with PBS plus 1% bovine serum albumin for 30 min at 37°C or overnight at 4°C. Threefold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 0.05% Tween 20 were then added for 1 h at 37°C or overnight at 4°C, and plates were washed three times with PBS plus 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM or IgG antibodies (200-ng/ml final concentration) in PBS plus 0.05% Tween 20 were then added, and plates were incubated at 37°C for 1 h. Plates were washed five times with PBS plus 0.1% Tween 20. Substrate (*p*-nitrophenylphosphate disodium; Sigma, St. Louis, MO) at 1 mg/ml in TM buffer (1 M Tris plus 0.3 mM $MgCl_2$, pH 9.8) was then added for 30 min at room temperature for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland). C polysaccharide (10 μg/ml) and PPS22F (20 μg/ml) were added into the serum dilution buffer for the anti-PPS3 ELISA in order to block PPS3-nonspecific Ig binding to the plate but were found to be unnecessary for the anti-PPS14 ELISA. Sera from R36A (unencapsulated variant of type 2 *S. pneumoniae*)-immunized mice also did not react in the anti-PPS14 ELISA.

Isolation and activation of peritoneal macrophages and spleen cells. RPMI 1640 (10 ml) was injected into the peritoneal cavity, through the fat pad, using a 23-gauge needle. After 3 min, lavage fluid containing the peritoneal cells was withdrawn using an 18-gauge needle. Cells were pelleted by centrifugation at $400 \times g$ for 10 min at room temperature. The cell pellet was suspended in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 50 μg/ml penicillin, and 50 μg/ml streptomycin) and adjusted to 1×10^6 total cells/ml, and 1 ml of the cell suspension was added to each well of a 24-well plate and incubated for 3 h at 37°C in a 5% CO_2-containing incubator. Plates were then washed to remove unbound cells. Fresh medium (0.5 ml) containing intact heat-killed Pn14 (1×10^7 CFU/ml) or CpG-ODN (2 μM) was added to the cultures of bound cells and incubated for 48 h at 37°C, followed by plate centrifugation and collection of culture supernatant (SN) for determination of cytokine concentrations by ELISA. Spleen cell suspensions were depleted of red blood cells using ACK lysing buffer (Quality Biological, Gaithersburg, MD) and suspended in culture medium at 5×10^6 cells/ml (1 ml/well in a 24-well plate) in the presence or absence of intact heat-killed Pn14 (1×10^7 CFU/ml) or CpG-ODN (2 μM) followed by 48 h of culture before removal of SN for determination of cytokine concentrations by ELISA.

Measurement of cytokine concentrations in culture supernatant. The concentrations of specific cytokines released into culture medium were measured using optimized standard sandwich ELISA. Recombinant cytokines used as standards as well as the capture monoclonal antibodies (MAbs), biotinylated MAbs used for detection, and streptavidin-alkaline phosphatase were purchased from Pharmingen BD. Streptavidin-alkaline phosphatase was used in combination with *p*-nitrophenyl phosphate disodium (Sigma, St. Louis, MO) as a substrate to detect specific binding. Standards were included in every plate, and the samples were tested in duplicate.

Bacterial protein extraction and $CD4^+$-T-cell priming assay. A frozen stock of Pn14 was thawed and subcultured on BBL premade blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Todd-Hewitt broth (Becton Dickinson, Sparks, MD) to mid-log phase (optical density at 650 nm of between 1.5 and 3.0). Pn14 protein was extracted with bacterial protein extraction reagent (Pierce, Rockford, IL). Spleen cell suspensions were depleted of red blood cells using ACK lysing buffer (GIBCO BRL). Spleen cells (1×10^7 cells) in 2 ml of medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 50 μg/ml penicillin, and 50 μg/ml streptomycin) were cultured in 24-well plates at 37°C in an atmosphere of 7% CO_2 and 95% humidity. After 30 min of incuba-

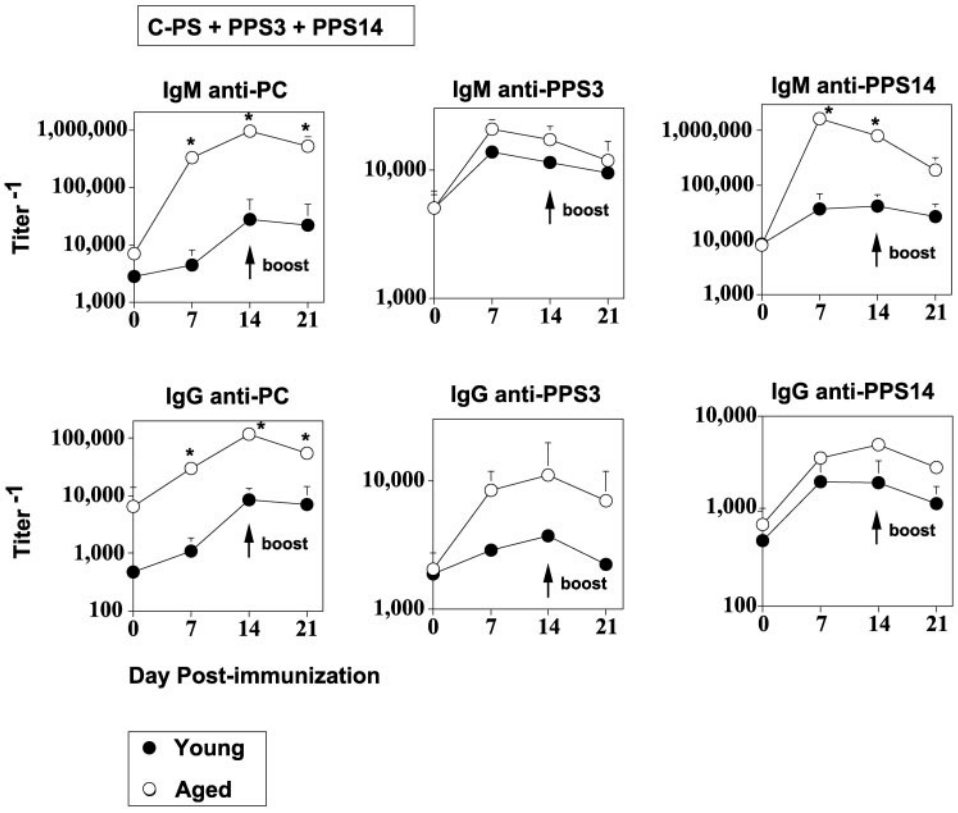

FIG. 1. In vivo IgG anti-PPS responses to isolated PPS preparations are similar or higher in aged mice relative to young adult mice. Aged and young adult mice (seven mice/group) were immunized i.p. with 1 μg each of PPS3, PPS14, and C-PS dissolved in sterile PBS. Mice were boosted on day 14, and sera were prepared from blood obtained from the tail vein on day 0 (before immunization), day 7, day 14, and day 21. Titers of IgM and IgG anti-PPS in serum were determined by ELISA. This experiment is representative of two independent experiments. Student's *t* test was used to determine significance: *A *P* value of ≤0.05 is considered statistically significant.

tion, 30 μg of Pn14 protein extract or recombinant PspA was added to each well. Following 24, 48, 72, or 96 h of culture, supernatants were collected for determination of released cytokine concentrations by ELISA. In selected experiments, prior to culture, spleen cells were depleted of $CD4^+$ T cells by magnetic cell sorting using anti-CD4-coated MicroBeads (Miltenyi Biotec, Auburn, CA) to establish that $CD4^+$ T cells were the source of the released cytokine. Magnetic cell sorting was performed according to the manufacturer's instructions using an LS MACS separation column (Miltenyi Biotec) with a MidiMACS magnetic separator. Cell purities were determined by flow cytometry.

Statistics. Titers of Ig in serum were expressed as geometric means of individual serum samples from seven mice ± standard errors of the means. Concentrations of secreted cytokines in culture SN were expressed as arithmetic means of duplicate cultures from macrophages or spleen cells pooled from 10 mice ± standard deviations. Levels of significance of the differences between groups were determined by the Student's *t* test. *P* values of ≤0.05 were considered statistically significant.

RESULTS

TI IgM and IgG responses to purified PPSs are equal or higher in aged mice relative to young mice. Aged mice exhibit defects in B-cell development and function (10, 43) as well as innate immunity (55). These abnormalities could lead to reduced TI responses to purified PS antigens (16, 51). To determine this, aged and young mice were immunized i.p. with a mixture of 1 μg each of C-PS, PPS3, and PPS14 in saline and boosted in a similar fashion 14 days later. Titers of IgM and IgG anti-PC, anti-PPS3, and anti-PPS14 in serum were determined separately by ELISA 0, 7, 14, and 21 days after primary immunization. A significant increase in titers of IgM and IgG anti-PC in serum was observed at all time points in aged mice relative to young adult mice ($P \leq 0.05$) (Fig. 1). Similarly, the IgM anti-PPS14 response in aged mice was significantly higher on days 7 and 14. Although IgM and IgG anti-PPS3 and IgG anti-PPS14 responses were also higher in aged mice than in young adult mice, the differences observed did not reach statistical significance. Collectively, these data indicate that in vivo TI responses to purified PPS antigens, and hence the responding B-cell population, are essentially intact in aged mice.

The anti-PPS14 response to a soluble PPS14-PspA conjugate in saline, but not intact Pn14, is highly defective in aged mice. The in vivo IgG anti-PPS14 response in young adult mice to a soluble conjugate of PPS14-PspA or to intact Pn14 is substantially higher than the IgG response to purified PPS14, based on the ability of the former immunogens to recruit $CD4^+$-T-cell help (26). In light of previous observations of defective $CD4^+$-T-cell function in aged mice (19, 52, 64, 67), we wished to determine whether the IgG anti-PPS14 responses to these TD forms of PPS14 would be affected relative to that observed in young adult mice. Thus, young adult and aged mice were immunized i.p. with either PPS14-PspA in saline (Fig. 2A) or intact, heat-killed Pn14 (Fig. 2B) and boosted 14 days later. Titers of IgG antiPPS14 and IgG anti-PspA in serum were determined separately by ELISA 0, 7, 14, 21, and

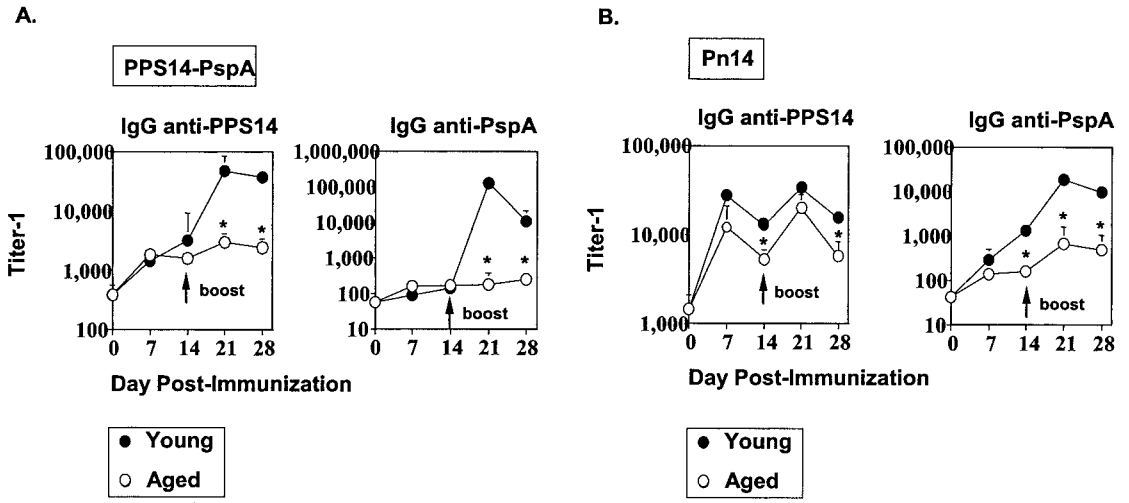

FIG. 2. In vivo IgG anti-PPS responses to PPS14-PspA in saline, in contrast to intact Pn14, are markedly defective in aged mice. Aged and young adult mice (seven mice/group) were immunized i.p. with 1 μg of PPS14-PspA in PBS or 2×10^8 CFU of heat-killed intact Pn14 in 200 μl of PBS. Mice were boosted on day 14, and sera were prepared from blood obtained from the tail vein on day 0 (before immunization), day 7, day 14, day 21, and day 28. Serum titers of IgG anti-PPS14 and IgG anti-PspA were determined by ELISA. This experiment is representative of two independent experiments. *A P value of ≤0.05 is considered statistically significant.

28 days after primary immunization. A weak but comparable primary induction of IgG anti-PPS14 was observed in both young adult and aged mice in response to PPS14-PspA in saline (Fig. 2A). In contrast, secondary immunization with PPS14-PspA led to a striking increase in IgG anti-PPS14 titers in young adult mice but virtually no secondary response in aged mice. Similarly, a strong secondary IgG anti-PspA response was observed in young adult mice but not aged mice. In contrast to soluble PPS14-PspA, intact Pn14 elicits a strong primary IgG anti-PPS14 response but consistently fails to elicit a boosted, secondary anti-PPS14 response (Fig. 2B) (25). In this regard, both the primary and secondary IgG anti-PPS14 responses to intact Pn14 were roughly comparable in young adult and aged mice, with only modest but significant reductions observed in aged mice on days 14 and 28 (Fig. 2B). In contrast, the primary and secondary IgG anti-PspA responses to intact Pn14 were strongly reduced in aged mice, although some boosting in the aged mice was observed following secondary immunization (Fig. 2B).

Using a depleting anti-CD4 MAb (GK1.5) (61) in vivo, we demonstrated that both the primary IgG anti-PPS14 response as well as the IgG anti-PspA response to intact Pn14 were strongly dependent on $CD4^+$ T cells in aged mice as well as young mice (Fig. 3). Collectively, these data indicate that the boosted, $CD4^+$-T-cell-dependent IgG anti-PPS14 (PPS14-PspA) and IgG anti-PspA (PPS14-PspA and intact Pn14) responses are markedly defective in aged mice. However, in response to intact Pn14, there is sufficient $CD4^+$-T-cell function to induce a strong primary IgG anti-PPS14 response in aged mice comparable to that observed in young adult mice.

CpG-ODN restores the secondary IgG anti-PPS14 response to PPS14-PspA in aged mice to young adult levels, with substantial, although partial, restoration of the IgG anti-PspA response. The capacity of intact Pn14, but not PPS14-PspA in saline, to elicit a strong, $CD4^+$-T-cell-dependent primary IgG

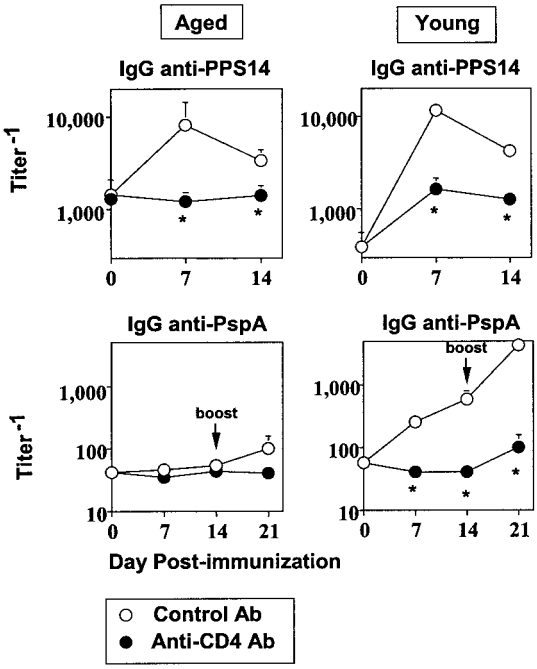

FIG. 3. In vivo IgG anti-PPS responses to Pn14 depends on $CD4^+$ T cells in both aged and young adult mice. One milligram of anti-CD4 MAb (Ab) (GK1.5) or control MAb (GL117) was injected i.p. into aged and young BALB/c mice (seven mice/group). One day later, mice were immunized i.p. with 2×10^8 CFU of heat-killed bacteria in 200 μl of PBS. Mice were boosted on day 14, and sera were prepared from blood obtained from the tail vein on day 0 (before immunization), day 7, day 14, and day 21. Titers of IgM and IgG anti-PPS14, anti-PC, and anti-PspA in serum were determined by ELISA. This experiment is representative of two independent experiments. *A P value of ≤0.05 is considered statistically significant.

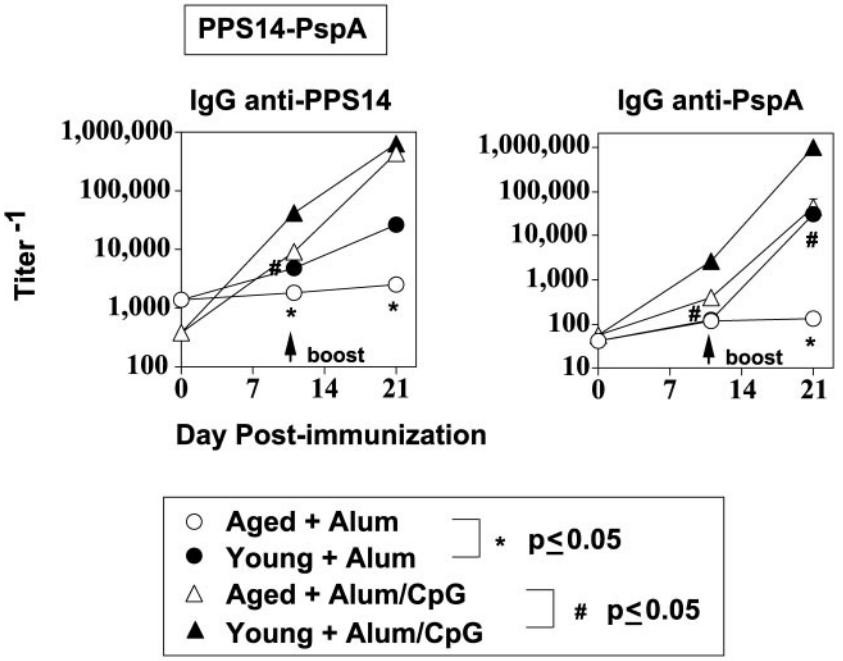

FIG. 4. CpG-ODN restores the in vivo IgG anti-PPS response and augments the IgG anti-PspA response to PPS14-PspA in aged mice. Aged and young adult mice (seven mice/group) were immunized i.p. with 1 μg of PPS14-PspA adsorbed onto 13 μg of alum or 1 μg of PPS14-PspA and 25 μg of CpG-DNA adsorbed onto 13 μg of alum. Mice were boosted on day 11, and sera were prepared from blood obtained from the tail vein on day 0 (before immunization), day 11, and day 21. Titers of IgG anti-PPS14 and IgG anti-PspA in serum were determined by ELISA. This experiment is representative of two independent experiments. * and #, P value of ≤0.05, which is considered statistically significant.

anti-PPS14 response in aged mice suggested the possibility that the expression of TLR ligands by the intact Pn14 might restore, at least partially, the defective $CD4^+$-T-cell function reported in the aged host (19, 20). Thus, we wished to determine whether the inclusion of a TLR ligand with PPS14-PspA could improve the $CD4^+$-T-cell-dependent IgG anti-PPS14 response in aged mice. PPS14-PspA was either adsorbed on alum alone or adsorbed on alum with an immunostimulatory unmethylated CpG-ODN, a ligand for TLR9 (21). Alum was added to optimize the effect of the CpG-ODN on the conjugate, but by itself, it had little, if any, adjuvanting effect relative to saline at the dosage used (data not shown). Young adult and aged mice were immunized i.p. and boosted on day 11. Similar to the response shown in Fig. 2A, the primary and secondary IgG anti-PPS14 responses to PPS14-PspA adsorbed on alum alone were barely detectable in aged mice, in contrast to those observed in young adult mice (Fig. 4). Inclusion of CpG-ODN resulted in significantly enhanced ($P < 0.05$) primary and secondary IgG anti-PPS14 titers in both aged and young adult mice relative to PPS14-PspA in alum alone (Fig. 4). Specifically, in contrast to the alum-alone group, we observed a detectable primary IgG anti-PPS14 response in the aged mice and a marked boost in titers following secondary immunization. Although the primary IgG anti-PPS14 titer observed in the group treated with alum plus CpG-ODN was still significantly lower in aged mice than in young adult mice, the boosted, secondary titers were equivalent.

Also, similar to the response shown in Fig. 2A, the secondary IgG anti-PspA response to PPS14-PspA on alum alone was barely detectable in aged mice (Fig. 4). In contrast, inclusion of CpG-ODN resulted in a striking enhancement in secondary IgG anti-PspA responses in both aged and young adult mice, although the titer in aged mice was still significantly lower than that observed in the young adult mice (Fig. 4). Thus, CpG-ODN essentially restores the IgG anti-PPS14 response to PPS14-PspA in aged mice to young adult levels, which is associated with a partial restoration in the IgG anti-PspA response. Coinjection of PPS14-PspA with an ODN negative control (GpC-ODN plus alum) that fails to activate TLR9 had no additional effect on the antibody response relative to the conjugate in alum alone (data not shown). This indicates that the adjuvanting effect of CpG-ODN was via TLR9 activation. Consistent with a recent report from our laboratory (48), the PPS14-PspA conjugate in saline was found to have some contaminating TLR-stimulating activity (data not shown), but as shown in Fig. 2A and 4, this was clearly insufficient by itself to restore Ig responses in the aged mice. A markedly defective primary and secondary IgG anti-PPS14 and anti-CRM197 response to the 7-valent pneumococcal conjugate vaccine, Prevnar, was also observed in aged mice relative to young adult mice (data not shown). Prevnar was purchased from Wyeth Pharmaceuticals (Philadelphia, PA) and consisted of seven PPS serotypes (serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) conjugated to diphtheria CRM197 protein and suspended in aluminum phosphate adjuvant (6). Prevnar also contains contaminating TLR-stimulating activity (48).

Aged and young adult mice secrete comparable amounts of proinflammatory cytokines from spleen cells and peritoneal macrophages in response to CpG-ODN, but aged cells release higher amounts of IL-10. To gain further insight into the

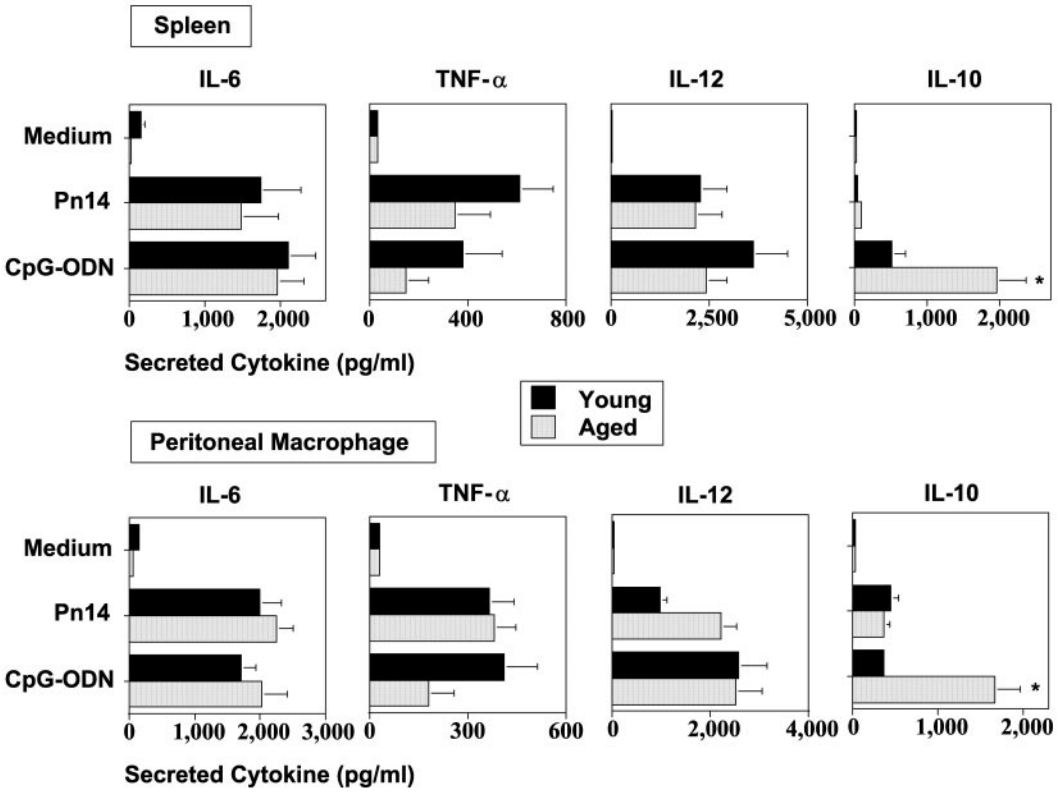

FIG. 5. Peritoneal macrophages and spleen cells from aged mice secrete amounts of proinflammatory cytokines similar to those from young adult mice but show increased levels of IL-10 upon stimulation with Pn14 or CpG-ODN. Peritoneal macrophages and spleen cells from naïve aged and young adult mice were incubated with 1×10^7 CFU of heat-killed Pn14 or 2 μM CpG-ODN for 48 h. Supernatants were collected for measurement of cytokine concentrations by ELISA. This experiment is representative of three independent experiments. *A P value of ≤ 0.05 is considered statistically significant. TNF-α, tumor necrosis factor alpha.

mechanism by which CpG-ODN acts as an effective adjuvant for anti-PPS14 and anti-PspA responses to PPS14-PspA in aged mice, we wished to determine its relative ability to induce pro- and anti-inflammatory cytokines. Thus, spleen cells and peritoneal macrophages from naïve young adult and aged mice were stimulated for 48 h in the absence or presence of CpG-ODN as well as intact Pn14, and the concentrations of interleukin-6 (IL-6), tumor necrosis factor alpha, and IL-12 (proinflammatory) as well as IL-10 (anti-inflammatory) were measured in culture SN by ELISA. Proinflammatory cytokine release by spleen cells and peritoneal macrophages in vitro in response to either intact Pn14 or CpG-ODN was essentially comparable in young adult and aged mice (Fig. 5). Of note, IL-10 secretion in aged spleen cells and peritoneal macrophages was significantly higher in response to CpG-ODN, but not intact Pn14, than that observed in young adult mice. These data suggest that the relatively normal release of proinflammatory cytokines in response to CpG-ODN in aged immune cells can account, at least in part, for its adjuvant effect, although this could be somewhat limited by concomitant increases in IL-10.

$CD4^+$-T-cell priming in response to PPS14-PspA in saline is defective in aged mice but can be augmented by the addition of CpG-ODN. The markedly reduced TD, but not TI, responses observed in aged mice strongly suggested that the underlying mechanism was defective $CD4^+$-T-cell help. Additionally, the ability of CpG-ODN to restore the IgG anti-PPS14 response to PPS14-PspA in aged mice further suggested that the CpG-ODN enhanced $CD4^+$-T-cell activation. Finally, we reasoned that intact Pn14, which expresses TLR ligands, might also induce substantial $CD4^+$-T-cell help in the aged mice. To investigate these possibilities, we first established an assay to measure $CD4^+$-T-cell priming in mice immunized with intact Pn14. Mice were immunized and boosted with intact Pn14, and spleen cells from naïve and Pn14-primed mice were obtained 14 days following the boost. An extract of purified pneumococcal proteins (PnP), obtained from Pn14, was added to spleen cell cultures for 1, 2, or 3 days, and the secretion of cytokines was measured by ELISA. The addition of PnP to spleen cell cultures obtained from Pn14-primed mice resulted in secretion of gamma interferon (IFN-γ) that was significantly higher ($P \leq 0.05$) than that obtained from unprimed spleen cell cultures (Fig. 6A). IFN-γ secretion from both primed and unprimed spleen cell cultures peaked between 48 and 72 h (Fig. 6A), with no detectable IFN-γ secretion observed in the absence of PnP (Fig. 6B). There was no detectable IL-4, IL-5, or IL-13 secretion at any of the time points in spleen cells from either primed or unprimed mice (data not shown). To confirm that $CD4^+$ T cells were the source of IFN-γ secretion in response to PnP, we selectively depleted $CD4^+$ T cells from primed spleen cell cultures. In contrast to whole spleen cells, $CD4^+$-T-depleted cells made no detectable IFN-γ in response to PnP (Fig. 6B). We next compared IFN-γ induction from

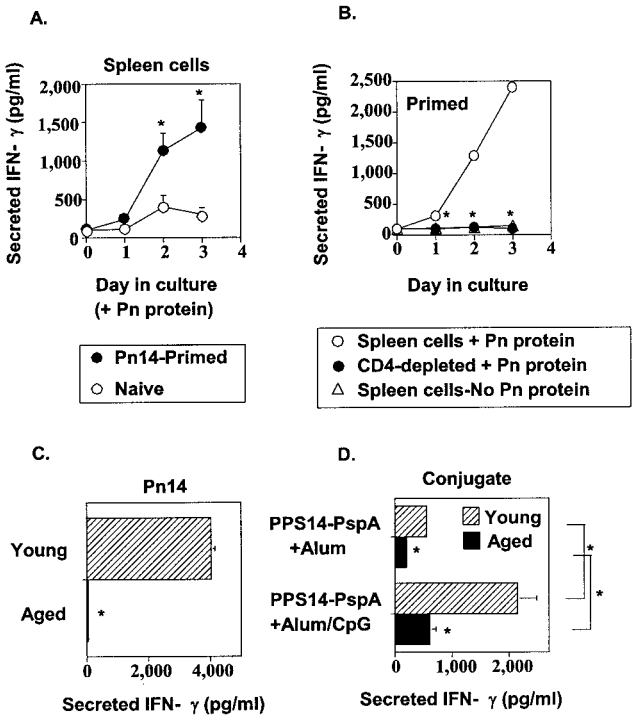

FIG. 6. CpG-ODN augments the defective $CD4^+$-T-cell priming in aged mice in response to PPS14-PspA. (A) Young adult mice were immunized i.p. with 2×10^8 CFU intact heat-killed Pn14 and boosted on day 14. On day 28, spleen cells were cultured at 1×10^6 cells/ml for 1, 2, or 3 days in the presence of 30 μg/ml of a pneumococcal protein extract. Spleen cells from naïve mice, used as controls, were similarly treated. Concentrations of secreted IFN-γ in culture SN were determined by ELISA. Data from one of five representative experiments are shown. (B) Spleen cells from Pn14-primed mice, with or without the depletion of $CD4^+$ T cells using magnetic bead sorting, were cultured as described above (A). Concentrations of secreted IFN-γ in culture SN were determined by ELISA. One experiment was performed. Aged and young adult mice were immunized with 2×10^8 CFU of heat-killed Pn14 and boosted on day 11 (C) or were immunized with 1 μg of PPS14-PspA adsorbed onto 13 μg of alum without or with 25 μg of CpG-DNA and boosted on day 11 (D). Six days later, spleen cell suspensions were prepared and incubated with 4 μg/ml of purified PspA for 72 h. Concentrations of secreted IFN-γ in culture supernatants were determined by ELISA. These experiments (C and D) are representative of two independent experiments. *A P value of ≤ 0.05 is considered statistically significant.

spleen cells taken from young adult and aged mice primed with Pn14. IFN-γ secretion from spleen cells derived from Pn14-immunized, aged mice was barely detectable in response to PspA (Fig. 6C) or PnP (data not shown), in contrast to that observed in their young adult counterparts, indicating a marked defect in $CD4^+$-T-cell priming. Thus, the expression of TLR ligands by intact Pn14 was not sufficient to induce detectable $CD4^+$-T-cell priming in the aged mouse. Likewise, spleen cells from aged mice immunized with PPS14-PspA on alum alone demonstrated significantly reduced IFN-γ secretion in response to purified PspA relative to that of young adult mice (Fig. 6D). However, inclusion of CpG-ODN with PPS14-PspA on alum resulted in significantly enhanced PspA-induced IFN-γ secretion in spleen cells from both aged and young adult mice, although IFN-γ secretion was still significantly higher in the spleen cells from the young adults (Fig. 6D). Thus, the inclusion of CpG-ODN resulted in an enhancement in $CD4^+$-T-cell priming in aged mice that correlated with the restoration of the IgG anti-PPS14 titers as shown in Fig. 4 as well as the increase in the IgG anti-PspA response. The higher levels of $CD4^+$-T-cell priming observed in young adult mice compared to those of aged mice through the inclusion of CpG-ODN likewise correlated with the higher IgG anti-PspA responses in the young adult host (Fig. 4).

DISCUSSION

These studies were designed to provide insight into the surprising and clinically relevant observation that in the elderly, as opposed to infants, TD IgG anti-PPS responses to Pn conjugate vaccine are not significantly better than TI IgG anti-PPS responses to PPS vaccine (1, 42). Thus, immunization with Pn conjugate vaccine is currently recommended for infants, whereas PPS vaccine is used for the elderly (>65 years of age) and immunocompromised adults (11). Nevertheless, it is a matter of dispute as to whether PPS vaccine provides significant protection in the elderly (4, 32), and as such, better vaccines are warranted for this at-risk population (42). In this regard, in this report, we demonstrated that whereas aged mice elicited comparable or higher IgG anti-PPS responses to TI PPS antigens relative to young adult mice, they were markedly defective in their TD IgG anti-PPS response to conjugate. This was associated with defective specific $CD4^+$-T-cell priming. Inclusion of CpG-ODN significantly boosted $CD4^+$-T-cell priming and restored the anti-PPS response to conjugate vaccine in the aged mice. Of note, in young adult mice, secondary IgG anti-PPS titers were substantially higher following immunization with conjugate in saline as opposed to purified PPS in saline, consistent with the ability of the former to recruit $CD4^+$-T-cell help (17). These data suggest that sufficient TLR-mediated adjuvanting of conjugate vaccine in the elderly might significantly boost production of protective IgG above that observed for PPS vaccine by restoring defective $CD4^+$-T-cell activation. In this regard, a recent study found no benefit of CpG-ODN in boosting TI IgG responses to purified PPS antigen in young adult mice (29), suggesting that a similar adjuvanting approach for PPS vaccine in the elderly will not be effective.

Our observation that PPS3 and PPS14 induce similar or even higher serum titers of IgM and IgG anti-PPS3 and anti-PPS14 in aged mice relative to young adult mice is consistent with several previous studies on induced Ig responses to PPSs as a function of age (50, 65). These data are also consistent with a number of reports in humans demonstrating comparable induction of various anti-PPS serotypes in elderly and young adult individuals in response to PPS vaccines (2, 22, 30, 38, 46). Of interest, anti-PPS titers did not correlate with opsonizing activity, and indeed, sera from elderly vaccinated individuals had lower pneumococcal opsonizing activity than sera from their younger counterparts (38). This might explain, in part, why PPS vaccines have demonstrated clear protective ability in young adults but not in the elderly (4, 32). In contrast, several reports have noted a moderate reduction in splenic plaque-forming cells specific for either PPS3 or the 23-valent PPS vaccine Pnu-Imune in

aged mice relative to young adult mice following PPS immunization (9, 16). Of interest, Pnu-Imune induced equal numbers of specific plaque-forming cells from mesenteric lymph nodes of aged and young adult mice (16), consistent with the finding of a relatively intact mucosal immune system in the aged host (56). Since we immunized mice by the i.p. route, B cells from the spleen and mesenteric lymph nodes would be expected to contribute to anti-PPS titers in serum. The striking increase in IgM and IgG anti-PC titers in aged mice relative to young adult mice following C-PS immunization is also consistent with a previous observation of increased frequencies of PC-specific B cells in aged BALB/c mice (66) and higher titers of anti-PC in sera from elderly individuals relative to young adult individuals vaccinated with different 23-valent PPS vaccine preparations (38). Collectively, these data support the notion of a relatively intact functional B-cell compartment in aged mice and humans that is potentially capable of eliciting robust Ig responses to PPS antigens, although perhaps with an altered qualitative profile.

TLR ligands are known to enhance anti-PS responses to conjugate vaccines (13, 31). The mechanism underlying this adjuvant effect is at least in part through the enhancement of antigen-presenting-cell function for T-cell activation (3, 29). In this regard, inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor alpha, typically induced by TLR ligands, can restore the defective IL-2 production in aged $CD4^+$ T cells, leading to improved T-cell function (20). Specifically, CpG-ODN has been shown to restore IgG responses to soluble proteins such as chicken ovalbumin (34) and diphtheria toxoid (35) in aged mice, including T-cell priming (34). The ability of innate immune cells from the aged host to secrete cytokines in response to TLR ligands has been studied extensively, with mixed results. As summarized previously by Kohut et al. (28), those studies, mostly using lipopolysaccharide as a stimulant, demonstrated both increased and decreased proinflammatory cytokine production in macrophages, depending to a large extent on their tissue source. In general, the cytokine responses of alveolar and splenic macrophages were found to increase with age, whereas those from peritoneal macrophages declined. In response to CpG-ODN, it was previously demonstrated that spleen cells from young adult and aged mice released comparable amounts of IL-12 (34).

Our observation that young adult and aged mice elicited roughly comparable TD IgG anti-PPS responses, although relatively poor IgG anti-PspA responses, to intact *S. pneumoniae* is consistent with the presence of TLR ligands within the bacterial cell wall. Surprisingly, this was still associated with poor $CD4^+$-T-cell priming. A previous observation of young adult mice indicated that optimal IgG anti-PPS responses to intact *S. pneumoniae* required a much briefer period of $CD4^+$-T-cell help and B7-dependent costimulation than that for IgG anti-PspA responses (26, 63). This suggests that even modest enhancements in $CD4^+$-T-cell activation may disproportionately increase anti-PPS as opposed to antiprotein responses. Furthermore, boosting with intact *S. pneumoniae*, in contrast to conjugate, did not result in a further enhancement of the IgG anti-PPS response in either young adult or aged mice. The major effect of CpG-ODN in boosting IgG anti-PPS responses to the conjugate in the aged mouse occurred following secondary immunization, and this was associated with a partial restoration of $CD4^+$-T-cell priming. Thus, our addition of CpG-ODN to the conjugate may have led to a more powerful adjuvanting effect than that exerted by the natural TLR ligands expressed by intact *S. pneumoniae*.

The relatively increased IL-10 production in cultures of spleen cells and peritoneal macrophages from aged mice in response to CpG-ODN is consistent with a general shift from type 1 to type 2 cytokine responses in the aged host (49). More specifically, lipopolysaccharide was previously found to stimulate the release of higher amounts of IL-10 from B cells (54) and macrophages (12) from aged mice and from monocytes derived from the elderly (45) relative to those obtained from the young host. IL-10 is generally considered an immunosuppressive cytokine (57). In this regard, we previously demonstrated that endogenous IL-10 inhibits in vivo antiphosphorylcholine responses to intact *S. pneumoniae* (27). Nevertheless, IL-10 has also been reported to enhance Ig responses to purified PPSs both in vitro and in vivo (33). The propensity of the aged host to release relatively higher levels of IL-10 may necessitate the use of stronger adjuvants in the elderly in order to overcome the increased immunosuppressive effects of this cytokine.

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